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(54) Title: METHOD AND KIT FOR LIGAND ASSAY

(57) Abstract: An assay of high sensitivity for substances present in low concentrations in body fluids is obtained when the product of immuno-PCR is subjected to capillary electrophoresis and preferably detected by laser-induced fluorescence.

"METHOD AND KIT FOR LIGAND ASSAY"

Field of the invention

This invention relates to a method of assay of a ligand, especially an antigen or hapten present in a low concentration in a body fluid. It also relates to a kit for this purpose.

Background of the invention

The *in vitro* assay of substances present in low concentrations in body fluids has traditionally been carried out by enzyme-linked immunosorbent assay (ELISA). Various methods of detection of the enzyme label have been used, of which enhanced chemiluminescent assay is one of the best. However, ELISA is not sensitive enough to detect very low concentrations. The polymerase chain reaction (PCR) has been coupled to the immunosorbent assay to provide a more sensitive assay. In this "immuno-PCR", a DNA oligomer is attached to the antibody in place of the enzyme label. The product of the reaction between an antigen or hapten to be detected and the antibody is thus attached, either directly or via a second antibody, to the DNA oligomer, which is then amplified by the PCR, thus producing a hugely amplified "signal" from the original reaction. The product of the PCR is then run on an agarose gel with an intercalating dye such as ethidium bromide to produce a visible signal. However, still more sensitivity is required from the assay in order to detect substances present in very low concentrations.

Summary of the invention

It has now been found that when the product of immuno-PCR is subjected to capillary electrophoresis, instead of conventional agarose gel electrophoresis, unexpectedly greater sensitivity can be obtained from the assay. This is further enhanced by laser-induced fluorescent detection of the product from capillary

electrophoresis, using a fluorescent intercalating dye.

Immuno-PCR was first described over 7 years ago by T. Sano *et al.*, Science *258*, 120-122 (1992). Capillary electrophoresis is also a technique practised for many years. It has not hitherto been appreciated that a combination of these two procedures would result in an immunoassay of exceptionally high sensitivity.

In one aspect, the invention provides a method of assay of an analyte ligand, which comprises amplifying a nucleic acid oligomer to which a specific binding partner of the analyte ligand is attached and determining the presence or amount of the amplified nucleic acid by capillary electrophoresis.

In another aspect, the invention provides a kit for carrying out a method of assay of the invention, comprising (1) a nucleic acid to which a specific binding partner of a ligand is attached, or the nucleic acid and binding partner components thereof, (2) coated capillaries and (3) a molecular sieve for inserting into the capillaries, preferably replaceably.

The term "assay", as used herein, means any qualitative, semi-quantitative or quantitative determination and thus covers both detection and measurement.

Brief description of the Figures

Figures 1 and 2 are plots of the fluorescent signal generated upon detection of the immuno-PCR products in capillary electrophoresis, by laser-induced fluorescence. Figure 1 relates to an assay of alpha-fetoprotein, described in Example 1. Figure 2 relates to assays of prion protein described in Example 2. The fluorescent signal on the y-axis is plotted against time on the x-axis.

Description of the preferred embodiments

The assay of the invention is applicable to any

ligand and a binding partner specific to that ligand, i.e. to any analyte to which ELISA can be applied. It is principally applied to antigens and haptens. (A hapten is a small molecule against which an antibody can be raised). It can also be applied to antibodies. Most preferably it is of interest for detecting antigens and antibodies present in body fluids, such as blood, urine, saliva, tears, nasal excretions and cerebrospinal fluid.

Since this assay is more time-consuming than ELISA, it is preferably applied to analytes that are diagnostic for rare diseases, such as prion proteins associated with transmissible spongiform encephalopathies in humans or animals. For this purpose, antibodies specific to the abnormal prion protein (PrP^{sc}) should be used. An example is 15B3, the preparation of which is described by C. Korth *et al*, Nature *390*, 74-77 (1997) and commercially available from Prionics AG, Zurich.

It may be performed in any of the formats well known for ELISA, but especially as a sandwich or an antigen or antibody capture assay.

Two particularly preferred formats are (a) those wherein the analyte ligand is an antigen or hapten and the assay comprises forming a sandwich between an immobilised first antibody to the antigen and a second antibody to the antigen, attaching the second antibody to a nucleic acid oligomer, amplifying the oligomer and determining the presence or amount of the amplified oligomer by capillary electrophoresis; and

(b) those wherein the analyte ligand is an antigen or hapten and the assay comprises immobilising the antigen, binding it to an antibody thereto, attaching this antibody to a nucleic acid oligomer, amplifying the oligomer and determining the presence or amount of the amplified oligomer by capillary electrophoresis.

The assay will usually be carried out in the normal

manner of an ELISA, a "universal" second antibody being used where appropriate. For example, if the analyte is a antigen and is captured on a microplate, a first antibody specific to it, especially a mouse monoclonal antibody, can be incubated with the antigen and, after washing, a further incubation is carried out with an anti-mouse "universal" second antibody. In ELISA, this second antibody is labelled with an enzyme. In immuno-PCR it is labelled with an oligonucleotide ("oligo").

In order to attach the oligo to the end antibody of the antigen/antibody detection "assembly", it is preferred to use a high affinity couple, especially biotin with either avidin or streptavidin, of which streptavidin is preferred because it is unglycosylated, minimising the possibility of side-reactions.

Most preferably, the oligo is biotinylated, i.e. biotin-labelled. It may contain uracil bases introduced deliberately to facilitate biotin-labelling. In principle, it can be of RNA or DNA. It is preferably double-stranded, so that it can be primed simultaneously with forward and reverse primers in the PCR.

Conveniently, the specific binding partner is biotinylated and the avidin or streptavidin group is attached bivalently to the biotinylated specific binding partner and the biotinylated oligo.

The oligo used for the immuno-PCR can be any arbitrary nucleic acid, so long as it does not self-anneal, or contain sequences which bind to proteins. It is preferably of DNA, most suitably non-human DNA, and preferably double-stranded. It may be of any appropriate length, but is preferably from 150 to 500bp, more preferably 180 to 350bp, most preferably from 200 to 300bp.

After the immuno-PCR has been carried out for a large number of cycles to amplify the product, the

product is preferably purified to eliminate low molecular weight products such as primers and primer-product dimers.

Other methods of amplification of the oligomer can be used instead of PCR. These include the ligase chain reaction and rolling circle amplification. For descriptions of PCR and the ligase chain reaction, see "Molecular Biology and Biotechnology" ed. Robert A Meyers, published by VCH Publishers, Inc., New York (1995) at pages 641-648 and 463-466 respectively. Rolling circle amplification is described in PCT Publication WO 97/19193.

The purified product is then subjected to an electrophoretic method which includes capillary flow of the buffer as well as molecular sieving. The molecular sieve matrix which is inserted into the capillary is preferably a hydrophilic polymer, especially a hydrophilic cellulosic polymer and most especially hydroxypropylmethylcellulose, which has been found to give better results in terms of signal to noise than cross-linked polyacrylamide. Other preferred hydrophilic polymers are hydroxyethylcellulose, methylcellulose, liquid agaraose (agar) or uncrosslinked polyacrylamide. Also usable, but less preferred, are polyvinyl alcohol, polyethylene oxide, polyethylene glycol, poly [(N-acryloylamino)ethoxyethanol], poly (N,N-dimethylacrylamide), poly [(N-acryloylaminoethoxy)ethyl-beta-glucopyranoside], poly (N-acryloylaminopropanol) and hydroxypropylcellulose. Application of a time-temperature gradient to the capillary electrophoresis, e.g. as disclosed in PCT Publication WO 96/08715, is not required and is therefore not preferred in this invention because it introduces unnecessary complexity.

The electrophoresis may be conducted in any vessel which provides for a thin layer of the molecular matrix,

so as to permit capillary action without electroosmotic flow (EOF) under an applied voltage. Preferably it is carried out in a capillary provided with a window to enable the laser light to be shone through it for the preferred laser-induced fluorescent (LIF) detection. The internal surface of the capillary is coated or treated to eliminate EOF.

In LIF, the dye is preferably one which detects the oligo by intercalation between the strands. Among suitable such dyes are those listed by Molecular Probes Inc. The laser is tuned to the wavelength of absorption of the dye, e.g. about 488 nm. Preferably the dye is pre-mixed with the sieving matrix and the resulting mixture inserted into the capillary as a plug. Additional dye can be added to the immuno-PCR product after purification, before electrophoresis.

Conveniently, the capillary electrophoresis is run with an internal standard of any arbitrary oligo which has a molecular weight well separated from that of the immuno-PCR product.

In the kit of the invention at least components (1) and (2) are provided in separate containers. Preferably the molecular sieve (3) is also provided separately from the capillaries (2), rather than the capillaries being pre-filled. The kit can include any of the other components preferred or illustrated in the Examples for the assay, as described herein.

The following Examples illustrate the invention. The words "Bluescript", "eCap", "GeneAmp", "GTG", "Nusieve", "Microcon", "Microseal", "Thermowell", "Tween", "Ultrafree" and "YOYO" are Trade Marks.

Example 1

Materials and Methods

Alpha-fetoprotein

Serum samples from patients affected with

hepatocarcinoma or testicular cancer, containing elevated levels of AFP, were used. Concentrations of AFP in the serum samples were pre-determined by ELISA and diluted as necessary to produce the lower concentrations. Each sample to be assayed was divided into two for comparative assay by the method of the invention and by immuno-PCR/agarose gel/UV light detection for comparison.

Biotin-labelled oligonucleotide synthesis

A 282bp length oligonucleotide was generated from a pBluescript II phagemid SK (+) *Sac* to *Kpn* (Stratagene, CA, USA), pBSII, using primers containing 20nt of a phagemid-specific sequence, and 20nt of a synthetic sequence ligated to it (underlined). The primers were pBSII1L (5′-biotin ATCGTTACGGCTATCCTTAGATTCAGGCTGCGCAACTGTT-3′, SEQ ID NO:1, containing nucleotides 469-488 of the phagemid and pBSII1R (5′-CCTAGGGTTACTAATCGTACGCAGGAATTCGATATCAAGC-3′ SEQ ID NO:2, containing nucleotides 710-691 of the phagemid. The PCR products will thus have a length of 20bp "left hand" synthetic sequence + 242bp phagemid sequence from nucleotides 469-710 + 20bp "right hand" synthetic sequence, total = 282bp. The synthetic sequences are substantially arbitrary

A 54µl PCR mixture containing 1mM $MgCl_2$, 0.5µM of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 0.5µM pBSII1L and 0.5µM pBSII1R, 0.01M Tris pH 8.3-55mM KCl and 0.5U r*Taq* polymerase (Pharmacia Biotech) was added to 6µl pBSII phagemid (2pg/µl). PCR amplification was carried out in a thermocycler (Perkin Elmer Gene Amp PCR System TC 9600). A sample was subjected to a PCR program starting with 10 PCR cycles, each with the following temperature profile: 30 seconds at 94°C, 5 seconds at 60°C with a decrease of 1°C/cycle until 50°C and 20 seconds at 72°C. Then the sample was subjected to 20

cycles, each with the following temperature profile: 30 seconds at 94°C, 5 seconds at 50°C and 20 seconds at 72°C followed by a final 10 min extension at 72°C. 10µl of the amplified PCR product, with 0.1% Bromophenol blue, were electrophoresed in a 2% agarose sieving gel of 1:1 agarose (Agarose ultra pure DNA, BioRad): "Nusieve" "GTG" Agarose (FMC Bioproducts, Rockland), stained with ethidium bromide, for 15 minutes at 150V. The agarose gel was photographed with a UV lamp at 260nm. 50µl of the amplified PCR product ("biotin-282") were diluted in 450µl of TBE buffer consisting of 89mM Tris-89mM borate-2mM EDTA pH 8.3 and purified three times by centrifugation through a Millipore "Microcon" 50 microconcentrator with a cut off at 50,000g. The filtrate was reconstituted in 50µl of TBE. The biotin-282 was then blotted as follows. 10µl of the thus purified sample were subjected to agarose gel electrophoresis, stained with ethidium bromide, photographed, and transferred onto a nylon membrane. A chemiluminescence blotting kit for peroxidase determination (Boehringer Mannheim) was used. The membrane was blocked with 10% blocking reagent (BM kit) in 0.1M maleic acid - 0.15M NaCl pH 7.5 (maleic buffer). A solution of 1/3000 peroxidase-labelled streptavidin (Dako), 0.33ppm in 5% blocking reagent in maleic buffer was added. By addition of the peroxide, as enzyme substrate, a single band corresponding to the biotin-labelled oligo was visualized following exposure of the membrane to X-OMAT S film (Kodak) and development. The purified samples were pooled and quantitated by measuring UV absorption at 260 nm, giving an optical density reading corresponding to 50µg/ml ds-DNA.

Sandwich Immuno-PCR

A 96-well polycarbonate microplate was coated

overnight at 4°C with 100µl of 1/74 polyclonal rabbit anti-human AFP (Dako), 15ppm in 0.1M sodium carbonate buffer pH 9.6. The plate was automatically washed 5 times with PBS buffer (15mM Na_2PO_4 - 120mM NaCl-2.7mM KCl, pH 7.4, Sigma). All subsequent washings were with fresh PBS buffer unless otherwise stated. Non-specific protein binding sites were blocked with 200µl carbonate buffer-4% BSA for 2h at 37°C. After washing the wells, 100µl of diluted AFP sample (analyte) were added and incubated for 2h at 37°C. After washing the wells, 100µl of 1/6120 mouse anti-human AFP monoclonal antibody (Sigma), 0.15ppm in PBS-1% BSA, were incubated for 1h at room temperature (R.T.) with shaking. After washing the wells, 100µl of 1/9400 biotin-labelled rabbit anti-mouse IgG (Dako), 1.5ppm in PBS-1% BSA, were incubated for 1h at R.T. with shaking. After washing the wells, 100µl of 1/500 streptavidin (2ppm in PBS) were incubated for 1h at 37°C. After washing the wells, the non-specific DNA-protein binding sites were blocked with 200µl PBS-2% BSA-0.1% herring sperm DNA (blocking buffer II) for 2h at 37°C. The blocking buffer II was prepared as follows. 10mg herring-sperm DNA (Boehringer Mannheim) in 1ml PBS was sonicated for 5 minutes, denatured for 10 minutes at 95°C and chilled quickly at 4°C. Then this herring-sperm DNA was added to 9ml of PBS containing 0.2gBSA, to prepare the blocking buffer II. After again washing the wells, 100µl of Biotin-282 (50zmol/µl in H_2O) were incubated for 30 min at R.T. without shaking. The microplate was automatically washed 10 times with distilled water. Then the wells were aspirated to dryness under vacuum. 2µl of water were added to each well thus prepared and to another well serving as negative PCR control. A positive PCR control well was filled with 2µl of Biotin-282 and another negative PCR mixture control well was left empty.

Then 18µl of PCR containing 1.5mM $MgCl_2$, 0.5µm of each dNTP, 2µm pBSII5L and 2µm pBSII5R, 0.01M Tris, pH 8.3-55mM KCl and 0.5U *rTaq* polymerase was added into each well. The primers were pBSII5L (5'-ATCGTTACGGCTATCCTTAG-3', SEQ ID NO:3) and pBSII5R (5'-CCTAGGGTTACTAATCGTAC-3', SEQ ID NO:4), which amplified the Biotin-282 sequence, the primers being of the respective synthetic sequences conatined in SEQ ID NO: 1 and 2 above. The microplate was sealed with a "Microseal" A system cap (MJ Research), a thermosensitive cap which needs a high temperature to seal the cap correctly on to the plate, and the plate was positioned onto the thermocycler with a spacer block (Costar) in order to keep the sealing cap tightly sealed to the plate. PCR amplification was carried out with the program described above. 10µl of the amplified PCR product with 0.1% Bromophenol blue were electrophoresed in the same way as for the biotin-282, to confirm the successful amplifications.

Purification of amplified product

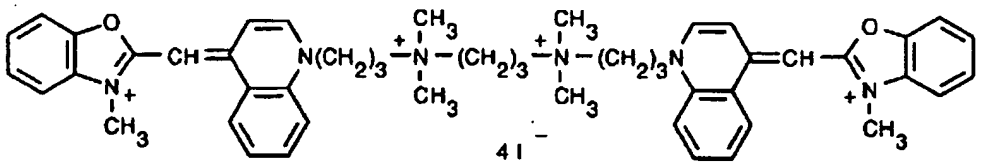
The remaining immuno-PCR product was purified to eliminate residual primers and primer-dimers. Using a Boehringer Mannheim kit, an 8µl sample of the immuno-PCR product, with 2µl of a 425bp oligo as internal standard, were mixed with 90µl TBE buffer to have a 100µl final volume. 500µl of binding buffer containing 3M guanidine thiocyanate, 10% Tris-HCl, 5% ethanol, pH 6.6 was added. The solution was spun at 13000g for 30 seconds through specially pre-treated glass fibre fleece. Nucleic acids having a minimum length of 100bp bind specifically to the fibre surfaces. The bound nucleic acids were then twice washed by spinning twice, each time for 30 seconds at 13000g with 500µl of 80% EtOH-20mM NaCl-2mM Tris-HCl, pH 7.5, in order to eliminate residual dimerised primers and excess primers. These low molecular weight nucleic acids

are not bound to the fibres and are therefore removed by this washing step. Then, nucleic acids of length at least 100bp were eluted from the glass fibres in a buffer of 10mM Tris-1mM EDTA, pH 8.5, with spinning for 30 seconds at 13000g. 100µl samples of the eluate were concentrated by centrifugation through a "Microcon" 10 microconcentrator with a cut off at 50,000g (Millipore, MA, USA) and 10µl of samples were ready to be analyzed by capillary electrophoresis.

Capillary Electrophoresis

Capillary electrophoresis was performed in a Beckman P/ACE System 5500 (Beckman, Fullerton, CA, USA) instrument equipped with an "eCap DNA" 47cm x 100µm i.d. coated fused silica capillary (Beckman). The capillary was made with a fused silica surface and an external polyimide coating, 365µm total diameter, to enhance its resistance. The interior surface of the capillary was presumably coated with a polyacrylamide network. The capillary was connected to the cathodic reservoir with the anodic reservoir connected to earth (electrically grounded). Each electrophoretic reservoir was filled with sieving buffer, described below. The electrophoresis was driven by an applied voltage through the capillary toward the anodic reservoir. On-column detection was by argon laser at 488nm excitation wavelenghts and 520nm emission wavelength through a 2mm glass window in the capillary, the window being without polyimide coating and present at 7cm from the anodic reservoir. Data were collected using an integrator, by the GOLD system purchased from Beckman. All buffer solutions were degassed immediately before use. Two pre-rinses, at 1.38 X 10^5 Pascal (20psi) pressure from a nitrogen tank, were performed: first for 5 min with sieving buffer (described below) and secondly for 12

seconds with 250nM of the fluorescent intercalating dye "YOYO-1 iodide" (Molecular probes, Oregon, USA) in TBE buffer. This dye has a high DNA binding affinity ("YOYO-1 iodide" has the molecular formula $C_{49}H_{58}I_4N_6O_2$ and the following structure:



All runs were performed at 20°C in a thermostatic environment. Immuno-PCR products were electrophoresed in 0.5% hydroxypropylmethylcellulose (HPMC for CE, Sigma) as sieving matrix in TBE buffer with 67nM YOYO-1 iodide, herein referred to as sieving buffer, at 200 V/cm (9.4KV) and 18μA for 30 minutes. The sample was injected into the cathodic end of the capillary by applying 3.45 X 10^3 Pascal (0.5psi) pressure for 12 seconds, after which the voltage was applied to cause migration of the DNA was toward the anode. Finally, the capillary was rinsed for 5 min with sieving buffer.

Laser-induced fluorescent detection

The fluorescent dye absorbs light at around 491nm and emits at about 509nm. Light is only absorbed when the dye is intercalated between the strands of ds DNA.

The emission of light was recorded and plotted on the y-axis as reference fluorescent units (RFU) against electrophoresis time in minutes on the x-axis.

Results

The results are shown in Fig. 1 where the AFP sample at 10^{-16}M is compared with the negative PBS control lacking any AFP analyte. Both curves gave peaks corresponding to the 425bp internal standard. A small peak indicated the presence of biotin-282 in the AFP sample. Similar plots for other concentrations of AFP

indicated that the limit of detection under the particular conditions is 10^{-16}M. This compares with 10^{-12} to 10^{-14} when the samples were assayed by immuno-PCR and immuno-PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide and detected by UV light at 260nm.

Example 2

Materials and Methods

Recombinant Prion protein rPrP

The mouse recombinant prion protein (rPrP) and polyclonal rabbit anti-mouse rPrP (R340) were kindly supplied by workers at the University of Zurich. Mouse recombinant prion protein can be prepared as described by S. Hornemann *et al.* FEBS Letters *413*, 277-281 (1997), Nature *390*, 74-77 (1997) at page 77 and is commercially available from Prionics AG, Zurich. Polyclonal antibodies thereto were raised in rabbits by a conventional technique. An alternative antibody, the monoclonal antibody 6H4 which recognises the normal and abnormal forms of prion proteins, as well as the recombinant form, is available from Prionics AG, Zürich. The polyclonal and monoclonal antibodies reacted with mouse, humans and hamster species.

ELISA (for comparison)

ELISA was carried out as described by K.-U.D. Grathwohl *et al*, Journal of Virological Methods *64*, 205-216 (1992). Briefly, a 96-well polystyrene microplate (NUNC) was coated with rPrP diluted in 3M guanidine thiocyanate in PBS (final pH<5). After incubation, washing and blocking the non specific binding site with PBS-3% BSA, either the 1/1000 polyclonal rabbit anti-rPrP (R340) (1 ppm) or the 1/2000 monoclonal anti-rPrP (6H4), diluted in blocking buffer, was applied onto the wells. After incubation and washing, the antigen-antibody complexes were visualized by the reaction between a

convenient phosphatase-labelled secondary antibody and para-nitrophenyl phosphate substrate. Colour development was measured with a microplate reader at a wavelength of 405nM.

Western blotting (for comparison)

Western blotting was carried out as follows. Diluted rPrP in PBS was prepared in denatured Laemmli buffer. rPrP samples were run on a 12% SDS-polyacrylamide gel and the proteins were transferred onto the PVDF membrane (BioRad) using a half-Towbin buffer of 25mM Tris - 192mM glycine - 10% MeOH. The PVDF membrane of a 1D-PAGE rPrP sample was stained with amido black and scanned. After blocking of non-specific binding membrane sites, antigen was recognized with primary polyclonal anti-rPrP (R340) 1/1000 or 7 ppm. Then a peroxidase-labelled secondary goat anti-rabbit antibody 1/1000 or 0.25 ppm (Dako) was used to label the antigen-antibody complexes. The peroxidase label was detected using enhanced chemiluminescence and X-ray films as described by the manufacturer of a kit for this purpose (Boehringer Mannheim).

Biotin-labelled oligonucleotide synthesis

A "biotin-282" ds DNA oligomer was prepared as in Example 1.

Spiked CSF sample

In one set of experiments the CSF samples were "spiked", as follows.

rPrP at different concentrations from 10^{-11} M to 10^{-19} M were added to cerebrospinal fluid from healthy volunteers. Then, each sample was diluted 10 times in 3M guanidine thiocyanate (GuaSCN) in PBS (15mM Na_2PO_4 - 120mM NaCl-2.7mM KCl pH 7.4, Sigma). The final range of concentrations was 10^{-12}M to 10^{-20}M of PrP in CSF. This "spiked CSF sample" was prepared so as to provide accurate concentrations of PrP, along with the "dirty

background" of a sample of CSF, so that the assay conditions are more representative of those encountered in clinical samples.

Indirect Immuno-PCR

A 96 well "Thermowell" microplate (Costar, NY, USA) made with a thin-wall polycarbonate surface, was coated overnight at 4°C with 100µl of either PrP spiked CSF sample or rPrP diluted in GuaSCN-PBS. The plate was automatically washed 5 times with PBS-0.05% "Tween" 20. Non-specific protein binding sites were blocked with 200µl PBS-3% BSA for 2h at room temperature (R.T.). After washing the wells, 100µl of 1/10,000 polyclonal rabbit anti-rPrP (0.1 ppmin PBS-3% BSA) were added and incubated for 2h at 37°C. After washing the wells, 100µl of 1/6,000 biotin-labelled goat anti-rabbit IgG (Sigma), 0.15 ppm in PBS-3% BSA, were incubated for 1h at R.T. with shaking. After washing, 100µl of 1/500 streptavidin (Sigma), 2 ppm in PBS, were incubated for 1h at 37°C. After washing the wells, the non-specific DNA-protein binding sites of the plate were blocked with 200µl of blocking buffer II (see Example 1) for 2h at 37°C. After again washing the wells, 100µl of biotin-282 (100z mol/µl in H_20) were incubated for 30 minutes at R.T.. The procedure thereafter was as described in Example 1.

Purification of amplified product

A centrifugal filter device, "Ultrafree-DA" (Millipore) was used to extract 100-10,000bp PCR product. 5µl of internal standard 425bp (home made) added to 10µl of each immuno-PCR sample were spun at 5,000g for 10 min. The filtrate was collected and was ready to be analyzed by CE-LIF.

Capillary electrophoresis

This was performed as in Example 1.

Results

ELISA and Western blotting analysis

In order to compare the sensitivity limit of different recombinant prion protein detection methods, ELISA and Western blotting were applied to rPrP detection. A series of samples of diluted rPrP in aqueous solution with a range of 10^{-7}M to 10^{-15}M with a step of one logarithmic unit between each were analyzed. The limit of rPrP detection was 0.9 X 10^{-9}M by ELISA. A guanidine thiocyanate buffer was used to dilute the rPrP in order to enhance protein interaction between the polystyrene microplate and the prion protein. A high improvement of signal to noise ratio was observed compared with using the conventional coating buffer, 0.1M carbonate buffer pH 9.6. The polyclonal antibodies gave more sensitive detection than the monoclonal, probably due to better antigen orientation against the antibody epitope. So, the polyclonal system was preferred for further experiments. Western blotting allows a similar limit of detection of rPrP (10^{-9}M) with the polyclonal system. Transmissible spongiform encephalopathies (TSEs) are associated with presence of an abnormal PrP called PrP^{sc}. PrP^{sc} is regarded as a sensitive and specific marker in both human and animal TSEs. PrP and PrP^{sc} differ only in a characteristic conformational change in their tertiary structure, without any principal modification in their primary amino acid sequence. To avoid risk of infection and to work in a safer environment, this method was carried out on normal recombinant mouse PrP.

Immuno-PCR

A background was observed due essentially to interactions between the biotin-labelled reagents, the polymeric surface of the plate and the antibodies. (Indeed, a higher background was observed with

polypropylene microplate as than a polycarbonate one.) A 10 times lower concentration of polyclonal antibodies (1/10,000 or 0.1 ppm) compared to that used in the ELISA (1/1000 or 1 ppm) eliminated the background. In parallel, a low concentration of biotin-labelled antibodies concentration did not enhance the background. A biotin-282 concentration of 100 zmol/µl involved no background with the agarose visualization of immuno-PCR products. A sensitivity limit of PrP in aqueous solution by such immuno-PCR was clearly 10^{-14}M and very slightly 10^{-16}M.

Purification of immuno-PCR products and capillary electrophoresis

Immuno-PCR products were analyzed by CE-LIF. Each immuno-PCR sample was purified before CE analysis to eliminate excess of primers using the most efficient and the most reproducible method. "Ultrafree-DA" from Millipore used in this example gave a higher reproducibility, with a sufficiently efficient purification, than the Boehringer Mannheim product used in Example 1. The latter gave better purification, due to a complete elimination of primer peaks: however, the reproducibility of this purification was lower and the procedure was more time-consuming.

After purification, immuno-PCR products were analyzed by CE-LIF. A sieving buffer containing a low concentration of hydroxypropylmethycellulose (HPMC) was successfully filled into the capillary to separate ds-DNA by CE-LIF. HPMC gave better LIF detection than the short chain non-cross linked polyacrylamide usually used for ds-DNA separation between 20 and 1000bp. It is believed that the high mobility of fragments in HPMC leads to rapid separations without loss of resolution compared with polyacrylamide. The sieving buffer contained the YOYO-1 iodide (see Example 1). A molar ratio of 5:1 DNA

bp: dye was applied to optimise the fluorescent intensity of the DNA. A small plug of TBE containing a relatively high concentration of YOYO-1 iodide (250nM) was filled into the column just before loading of sample. It was found best to mix the HPMC with the YOYO-1 iodide and then fill the capillary with the mixture. Since only biotin-282bp and the internal standard (425bp) have to be separated, a capillary length of 37cm (instead of 47cm) can be used in order to migrate sample for only 20 min instead of the 30 min of Example 1. A comparison of each sample electropherogram (Fig. 2) showed the biotin-282bp peak increasing with the amount of PrP in sample. A background was observed on each blank sample due to the high sensitivity of LIF detection. However, a limit of PrP detection was observed at 10^{-16}M. A 100 fold enhancement of sensitivity was shown compared with the immuno-PCR method using agarose gel analysis and a 10^7 fold enhancement in sensitivity compared with conventional ELISA. It is confidently expected that this signal to noise ratio will be enhanced without loss of sensitivity by decreasing the amount of Biotin-282 in each well.

With the spiked CSF samples, PrP was also detected by immuno-PCR/CE-LIF at very low concentrations.

* * *

Each of the above-mentioned references is herein incorporated by reference to the extent to which it is relied on herein.

CLAIMS

1. A method of assay of an analyte ligand, which comprises amplifying a nucleic acid oligomer to which a specific binding partner of the analyte ligand is attached and determining the presence or amount of the amplified nucleic acid by capillary electrophoresis.

2. A method according to Claim 1, wherein the oligomer is biotinylated and the specific binding partner has an avidin or streptavidin group attached to it.

3. A method according to Claim 2, wherein the specific binding partner is biotinylated and the avidin or streptavidin group is attached bivalently to the biotinylated specific binding partner and the biotinylated oligomer.

4. A method according to Claim 1, 2 or 3, wherein the analyte ligand is an antigen or hapten and the assay comprises forming a sandwich between an immobilised first antibody to the antigen and a second antibody to the antigen, attaching the second antibody to a nucleic acid oligomer, amplifying the oligomer and determining the presence or amount of the amplified oligomer by capillary electrophoresis.

5. A method according to Claim 1, 2 or 3, wherein the analyte ligand is an antigen or hapten and the assay comprises immobilising the antigen, binding it to an antibody thereto, attaching this antibody to a nucleic acid oligomer, amplifying the oligomer and determining the presence or amount of the amplified oligomer by capillary electrophoresis.

6. A method according to any preceding Claim, wherein the nucleic acid oligomer is amplified by an immuno-PCR procedure.

7. A method according to any preceding Claim, wherein the capillary electrophoresis is carried out in a molecular sieve of particles of a cellulose polymer.

8. A method according to Claim 7, wherein the particles are of hydroxypropylmethylcellulose.

9. A method according to any preceding Claim wherein the oligomer has a length of 150 to 500bp.

10. A method according to any preceding Claim, wherein the determination of the presence or amount of the amplified nucleic acid in the capillary electrophoresis is carried out by laser-induced fluorescence detection in the presence of a fluorescent intercalating dye.

11. A kit for carrying out a method as defined in Claim 1, comprising (1) a nucleic acid to which a specific binding partner of a ligand is attached, or the nucleic acid and binding partner components thereof, (2) coated capillaries for capillary electrophoresis and (3) a molecular sieve for inserting into the capillaries.

12. A kit according to Claim 11, wherein the nucleic acid is biotinylated.

13. A kit according to Claim 11 or 12, wherein the oligomer is biotinylated and the specific binding partner has an avidin or streptavidin group attached to it.

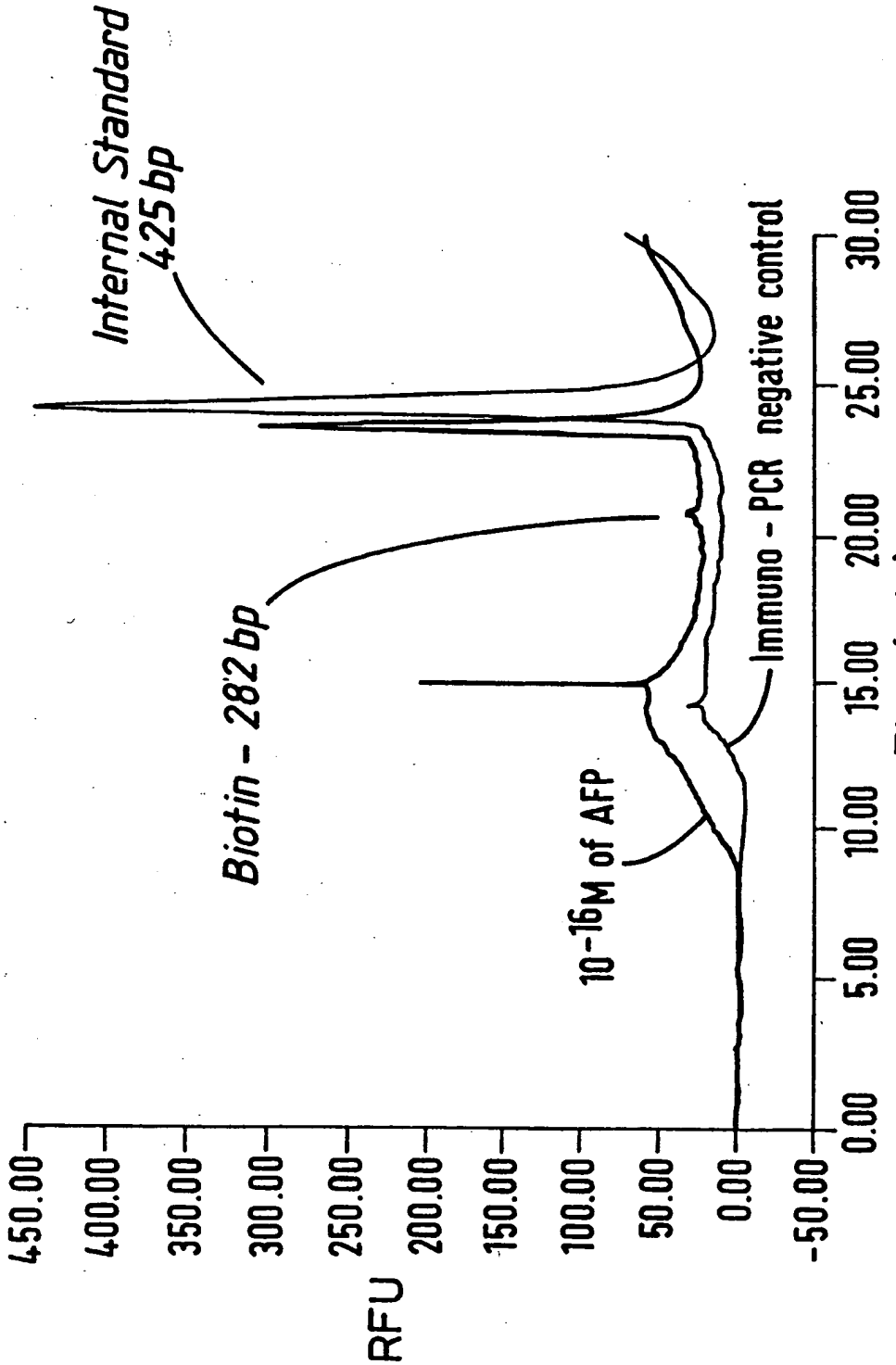
14. A kit according to Claim 13, wherein the specific binding partner is biotinylated and the avidin or streptavidin group is attached bivalently to the biotinylated specific binding partner and the biotinylated oligomer.

15. A kit according to Claim 14, wherein the molecular sieve comprises a cellulose polymer or a gel polymer.

16. A kit according to Claim 15, wherein the cellulose or gel polymer is hydroxypropylmethylcellulose.

17. A kit according to any one of Claims 11 to 16, wherein the oligomer has a length of 150 to 500bp.

18. A kit according to any one of Claims 11 to 17, further containing a fluorescent intercalating dye.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Biotin - 282 bp
10-16M of AFP
Immuno - PCR negative control
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400.00
350.00
300.00
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150.00
100.00
50.00
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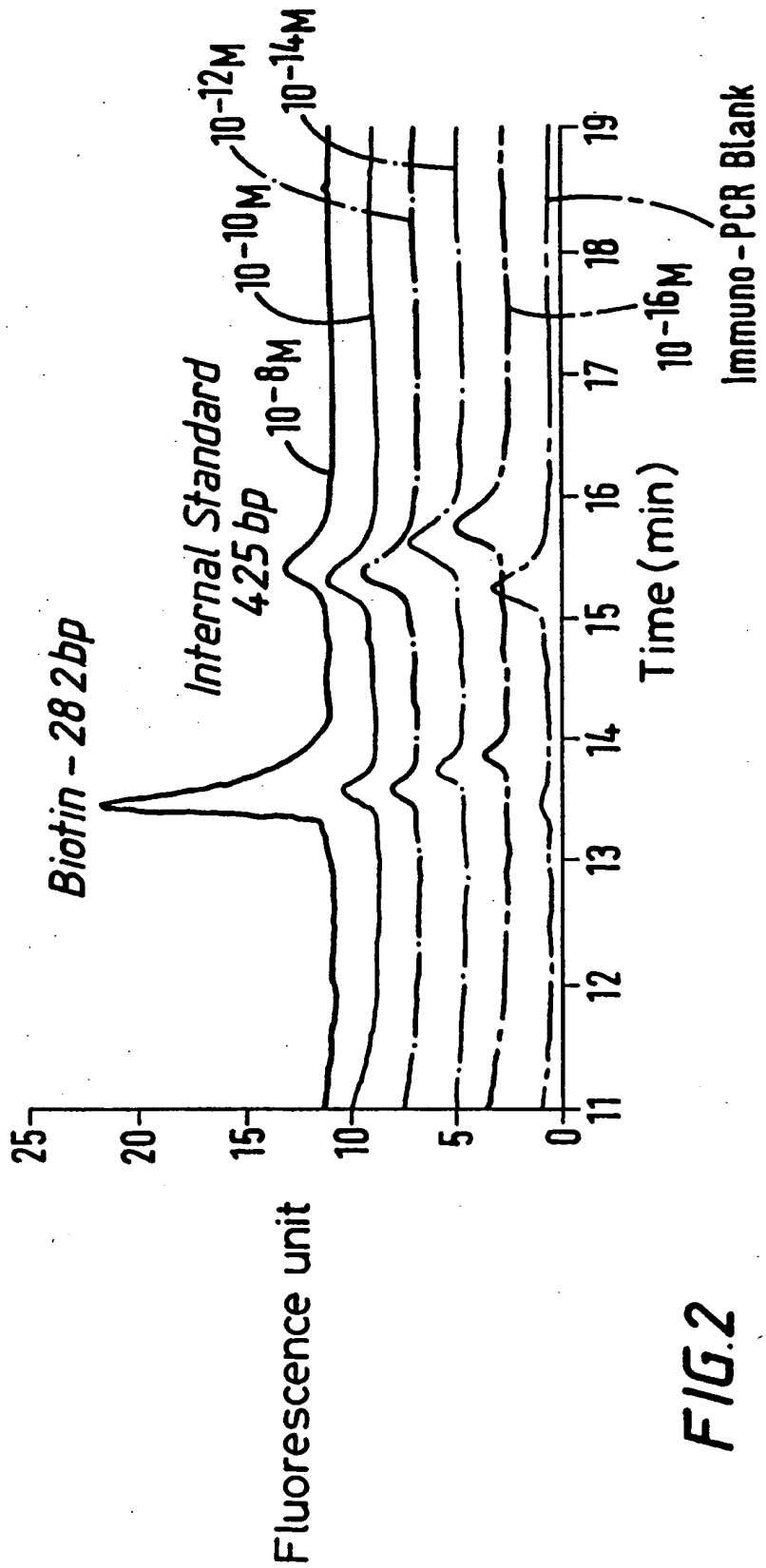
25.00
30.00
Time (min)

FIG.1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Internal Standard
425 bp
10-8M
10-10M
10-12M
10-14M
10-16M
Immuno-PCR Blank
Fluorescence unit
Time (min)
25
20
15
10
5
0
11
12
13
14
15
16
17
18
19

FIG.2

SEQUENCE LISTING

<110> UNIVERSITE DE GENEVE
Hochstrasser, Denis F
Sanchez, Jean-Charles
Zimmermann, Catherine G

<120> Method and kit for ligand assay

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<140>
<141>

<150> GB9912743.3
<151> 1999-06-02

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<223> Description of Artificial Sequence: PCR primer consisting of phagemid sequence contained in SEQ ID NO: 2

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INTERNATIONAL SEARCH REPORT

Int. Ional Application No
PCT/EP 00/04381

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/543 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	22/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Reuter, U

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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2

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/EP 00/04381

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 08715 A (RIGHETTI PIER GIORGIO ;GELFI CECILIA (IT)) 21 March 1996 (1996-03-21) cited in the application the whole document	1-18
Y	FASCO M J ET AL: "QUANTITATIVE RNA-POLYMERASE CHAIN REACTION-DNA ANALYSIS BY CAPILLARY ELECTROPHORESIS AND LASER-INDUCED FLUORESCENCE" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 224, no. 1, 1995, pages 140-147, XP000486747 ISSN: 0003-2697 the whole document	1-18
A	WO 98 53100 A (ANGELETTI P IST RICHERCHE BIO ;MONACI PAOLO (IT); CORTESE RICCARDO) 26 November 1998 (1998-11-26) page 2-3 page 9	1-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/EP 00/04381

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